

AD\_\_\_\_\_

Award Number: W81XWH-05-1-0357

TITLE: Mutations in PUMA Gene Cause Prostate Cancer Development and Agressiveness

PRINCIPAL INVESTIGATOR: Chien-An Hu, Ph.D.

CONTRACTING ORGANIZATION: The University of New Mexico  
Albuquerque, NM 87131

REPORT DATE: November 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE   |             |                         |                            | Form Approved<br>OMB No. 0704-0188                       |   |
|---|-------------|-------------------------|----------------------------|--|---|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>   |             |                         |                            |  |   |
| 1. REPORT DATE (DD-MM-YYYY)<br>01-11-2006   |             | 2. REPORT TYPE<br>Final |                            | 3. DATES COVERED (From - To)<br>1 MAY 2005 - 31 OCT 2006 |   |
| 4. TITLE AND SUBTITLE<br>Mutations in PUMA Gene Cause Prostate Cancer Development and Aggressiveness  |             |                         |                            | 5a. CONTRACT NUMBER                                      |   |
|   |             |                         |                            | 5b. GRANT NUMBER<br>W81XWH-05-1-0357                     |   |
|   |             |                         |                            | 5c. PROGRAM ELEMENT NUMBER                               |   |
| 6. AUTHOR(S)<br>Chien-An Hu, Ph.D.<br><br>E-mail: AHU@SALUD.UNM.EDU   |             |                         |                            | 5d. PROJECT NUMBER                                       |   |
|   |             |                         |                            | 5e. TASK NUMBER  |   |
|   |             |                         |                            | 5f. WORK UNIT NUMBER                                     |   |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>The University of New Mexico<br>Albuquerque, NM 87131   |             |                         |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER                 |   |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012   |             |                         |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)                         |   |
|   |             |                         |                            | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)                   |   |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br>Approved for Public Release; Distribution Unlimited  |             |                         |                            |  |   |
| 13. SUPPLEMENTARY NOTES   |             |                         |                            |  |   |
| 14. ABSTRACT<br>In the first year of this project, we surveyed the PUMA gene expression using RT-PCR amplification of RNA isolated from three PCa cell models, LNCaP, DU145 and PC3, BPH (benign prostate hyperplasia) cells or normal prostate tissues. Our results showed that expression of PUMA was relatively high in normal prostate tissues/cells but was significantly reduced in two PCa cell models, DU145 and PC3. In addition, to survey the PUMA gene for mutations using PCR amplification of genomic DNA and direct sequencing of amplified amplicons, we have successfully developed PCR protocols to amplify all three PUMA coding exons and confirmed the amplified sequences were <i>bona fide</i> PUMA. Furthermore, during the course of study, we initiated a new collaboration with Dr. Stephen Thibodeau, Mayo Clinic, Rochester MN, to obtain de-identified clinical samples which were isolated from the PCa patients with the association between chromosome 19q13 and PCa aggressiveness (1). Drs. Thibodeau and Schaid (PI's another collaborator) have been collaborating for many years and have been making outstanding contributions in PCa research (1-5). Finally, we started surveying the PUMA gene for mutations using PCR amplification of genomic DNA and direct sequencing of amplified amplicons of the clinical samples that were isolated from PCa patients and controls mentioned above. |             |                         |                            |  |   |
| 15. SUBJECT TERMS<br>PUMA, prostate cancer (PCa), gene mutation, PCR, reverse transcriptase PCR (RT-PCR), exon  |             |                         |                            |  |   |
| 16. SECURITY CLASSIFICATION OF:   |             |                         | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES                                      | 19a. NAME OF RESPONSIBLE PERSON           |
| a. REPORT   | b. ABSTRACT | c. THIS PAGE            |                            |  | USAMRMC                                   |
| U   | U           | U                       | UU                         | 11   | 19b. TELEPHONE NUMBER (include area code) |

## Table of Contents

|  | PAGE        |
|--|-------------|
| <b>Cover.....</b>                        | <b>1</b>    |
| <b>SF 298.....</b>                       | <b>2</b>    |
| <b>Introduction.....</b>                 | <b>4</b>    |
| <b>Body.....</b>                         | <b>4-7</b>  |
| <b>Key Research Accomplishments.....</b> | <b>7</b>    |
| <b>Reportable Outcomes.....</b>          | <b>4-6</b>  |
| <b>Conclusions.....</b>                  | <b>6</b>    |
| <b>References.....</b>                   | <b>6-7</b>  |
| <b>Appendices.....</b>                   | <b>8-11</b> |

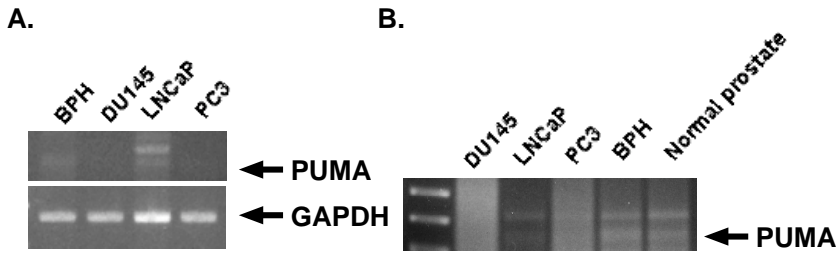
## Introduction

As we stated in the proposal, based on our preliminary observation that expression of PUMA (P53 Upregulated Modulator of Apoptosis) is relatively high in normal prostate cells and significantly reduced in prostate cancer (PCa) cell models, and the fact that PUMA's chromosome localization on 19q13 and PUMA's role as a pro-apoptotic effector, we hypothesized that PUMA may be a candidate gene (or susceptibility locus) that, when mutated or downregulated, promotes tumor progression and aggressiveness. Moreover, based on the strong familial linkage between chromosome 19q13 and PCa in different families, and the availability of DNA samples from PCa patients, we further hypothesized that there may be some pathological point-mutation or small-deletion mutant alleles of PUMA that were inherited in the chromosome 19q13-associated PCa families. We proposed three tasks in this proposal: *Task 1.* To survey the PUMA gene for mutations using RT-PCR amplification, PCR amplification of genomic DNA and direct sequencing of amplified amplicons from PCa cell models, LNCaP, DU145 and PC3 (Months 1-12). *Task 2.* To survey the PUMA gene for mutations using PCR amplification of genomic DNA and direct sequencing of amplified amplicons of the clinical samples that were isolated from PCa patients and control (Months 1-18). *Task 3.* To investigate the functional consequences of point mutations or small deletions in PUMA, if any, in PUMA-induced apoptosis (Months 6-18).

**In the first year of this project,** we surveyed the PUMA gene expression using RT-PCR amplification of RNA isolated from three PCa cell models, LNCaP, DU145 and PC3, BPH (benign prostate hyperplasia) cells or normal prostate tissues. **Our results showed that expression of PUMA was relatively high in normal prostate tissues/cells but was significantly reduced in two PCa cell models, DU145 and PC3.** In addition, to survey the PUMA gene for mutations using PCR amplification of genomic DNA and direct sequencing of amplified amplicons, **we have successfully developed PCR protocols to amplify all three PUMA coding exons and confirmed the amplified sequences were *bona fide* PUMA.** Furthermore, during the course of study, **we initiated a new collaboration with Dr. Stephen Thibodeau, Mayo Clinic, Rochester MN, to obtain de-identified clinical samples which were isolated from the PCa patients with the association between chromosome 19q13 and PCa aggressiveness (1).** Drs. Thibodeau and Schaid (PI's another collaborator) have been collaborating for many years and have been making outstanding contributions in PCa research (1-5). Finally, **we started surveying the PUMA gene for mutations using PCR amplification of genomic DNA and direct sequencing of amplified amplicons of the clinical samples that were isolated from PCa patients and controls mentioned above.**

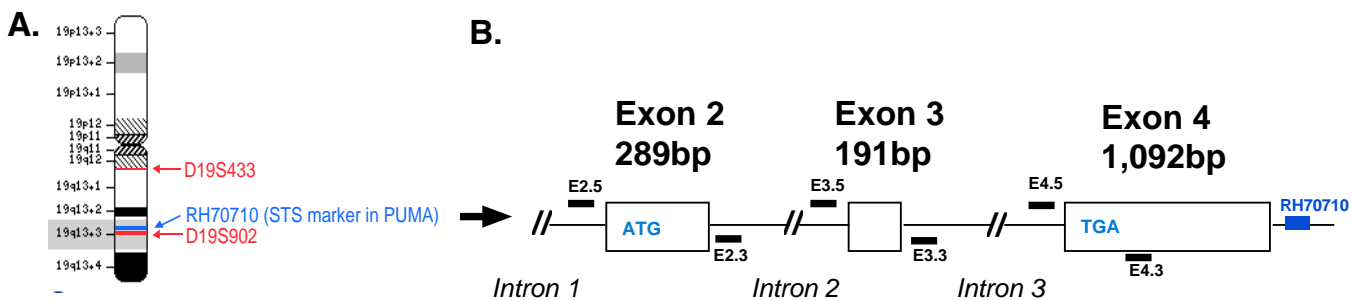
## Reportable Outcomes

**A. Analysis of the expression of PUMA in three PCa model cell lines by RT (reverse transcriptase)-PCR** To study the expression of PUMA in PCa cells, BPH cells or normal prostate tissues at the RNA level, we designed two exonic primers, PUMA.5 (bp +220 - 236; 5'-GGTCCCCGCTGGCCTGG-3') and PUMA.3 (bp +594 - 572; 5'-GGGTGCAGGCACCTAATTGG-3') to conduct RT and subsequent PCR using a standard protocol (6). We have successfully amplified the corresponding (~two third of) coding sequence of the PUMA cDNA by RT-PCR (Fig. 1). Importantly, our results during the first year of the funding period showed that expression of PUMA was relatively high in normal prostate tissues/cells but was significantly reduced in two PCa cell models, DU145 and PC3 (Fig. 2A&B). We are in the process of investigating the molecular bases of downregulation of PUMA expression in DU145 and PC3 cells.

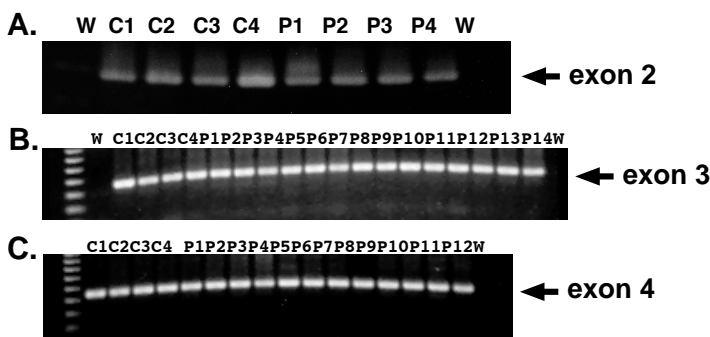


**Figure 1, Expression of PUMA is downregulated in two of the PCa model cell lines, DU145 and PC3.** Basal expression of PUMA is detected in BHP cells and normal prostate tissues. (A) and (B) are two independent experiments. BPH, benign prostate hyperplasia cells.

**B. Amplification and sequencing of the three coding exons of PUMA** To investigate whether or not PUMA gene is mutated in its coding sequence of PCa patients, we first set out to amplify the coding exons (exon 2, 3, 4) of human PUMA gene using control genomic DNA. Practically, there was a technical challenge: all 3 of the coding exons of PUMA gene are high GC rich [GC content: exon 2, 82.35% (238/289); exon 3, 76.4% (146/191); exon 4, 61.7% (37/60)]. After comparing and contrasting many PCR protocols and primer sets (Figure 2), we finally PCR amplified all three coding exons of PUMA gene from DNA isolated from both control and patient samples successfully (Figure 3). Direct sequencing analysis confirmed that all PCR products of control DNA possess expected wildtype (wt) PUMA sequence. In addition, we conducted direct sequencing of amplified DNA samples of exons 3 and 4 from the clinical samples of 14 PCa patients (Figure 3 B and C). So far, we did not find mutations in those two exons of PCa patients (data not shown). We are in the process of sequencing amplified exon 2 sequences of the 14 PCa patients and will amplify and sequence exons 2-4 of the other PCa DNA samples.



**Figure 2, (A). Ideogram of human chromosome 19.** Three chromosome 19 markers, D19S902, D19S433 and RH70710, and the location of PUMA structural gene were indicated. The distance between D19S902 and D19S433 is 20 centiMorgan. RH70710, a STS marker in the 3' untranslated region of the exon 4 of PUMA gene, is 600 kb centromeric to D19S902. **(B). The structural gene of Human PUMA.** Size of each exon and locations of primers used in PCR are as indicated.



**Figure 3. Successful amplification of exons 2, 3 and 4 of PUMA gene from genomic DNA isolated from both control and PCa patients.** (A). amplification of exon 2 from 4 control samples and 4 PCa patients. (B). amplification of exon 3 from 4 control samples and 14 PCa patients. (C). amplification of exon 4 from 4 control samples and 12 PCa patients. W, water only; C, DNA isolated from control clinical sample; C1 to C4, DNA isolated from 4 independent control prostates; and P1 to P14, 14 independent DNA samples isolated from prostate tumors.

## Conclusions

Loss of PUMA either by mutations or allelic imbalance could play a key role in cancer cell invasion and metastasis and develop into more of an “anti-apoptosis genotype” as aggressiveness and metastasis progress. Our idea of investigating mutations of PUMA in 19q13-associated PCa patients and likelihood of PUMA functioning as a tumor suppressor gene is innovative. Recently, we showed that PUMA overexpression induces ROS generation and proteasome-mediated stathmin degradation in colorectal cancer cells (6), again, demonstrating the importance of PUMA in cancer apoptosis. Our findings in this project may offer potential groundbreaking concepts and therapeutic strategies in prostate cancer.

To investigate whether or not the PUMA gene is mutated in 19q13-associated PCa patients, we have been collaborating with Drs. Dan Schaid and Stephen Thibodeau at Mayo Clinic, Rochester, MN, for conducting mutation analysis on de-identified DNA samples isolated from the PCa patients that showed familial association between chromosome 19q13 and PCa. We also have been collaborating with Dr. Jeffrey Griffith, Professor and Chair, Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, who has DNA samples from ~100 archival prostate tumors, 160 benign prostate biopsies, 312 malignant prostate biopsies and 377 specimens of matched tissues from prostatectomy. We have started surveying the PUMA gene for mutations using RT-PCR amplification, PCR amplification of genomic DNA and direct sequencing of amplified amplicons. We have successfully amplified the two third of the coding sequence of the PUMA cDNA by RT-PCR (Fig. 1). We also have successfully PCR amplified all three coding exons, exon 2, 3, 4, of PUMA gene from DNA isolated from both control and patient samples (Figure 3). Direct sequencing analysis confirmed that all PCR products of control DNA possess expected PUMA sequence. In addition, we conducted direct sequencing of amplified DNA samples of exons 3 and 4 from the clinical samples of 14 PCa patients (Figure 3 B and C). So far, we did not find mutations in those two exons of PCa patients (data not shown). We will sequence the exon 2 amplicons of the 14 PCa patients and will amplify and sequence exons 2-4 of the other PCa DNA samples in the coming funding period.

If any point mutations or small deletions in PUMA coding exons are found, functional consequences of the mutant alleles will be conducted. Previously, we have constructed adenovirus (AD) harboring full-length wt PUMA cDNA (AD-PUMA) and shown that overexpression of PUMA by AD-PUMA induces marked BAX- and mitochondria-mediated apoptosis in colorectal cancer cells, DLD-1, whereas, AD-PUMA possessing a BH3 domain deletion (AD-PUMA-dBH3) completely abolishes its activity to induce apoptosis (6). Using the same approach, we will construct PUMA mutant alleles in AD and conduct apoptosis assay in DLD-1 cells. Pathological loss-of-function mutations of PUMA cDNA should not induce apoptosis. In contrast, PUMA polymorphic alleles, like wt PUMA, should be able to induce apoptosis. Using the same approach, we will construct PUMA mutant alleles in AD and conduct apoptosis assay in DLD-1 cells. Pathological loss-of-function mutations of PUMA cDNA should not induce apoptosis. In contrast, PUMA polymorphic alleles, like wt PUMA, should be able to induce apoptosis.

## References

1. Slager SL, Schaid DJ, Cunningham JM, McDonnell SK, Marks AF, Peterson BJ, Hebbbring SJ, Anderson S, French AJ, Thibodeau SN. (2003) Confirmation of linkage of prostate cancer aggressiveness with chromosome 19q. *Am J Hum Genet.* 72:759-62.

2. Narla G, Difeo A, Reeves HL, Schaid DJ, Hirshfeld J, Hod E, Katz A, Isaacs WB, Hebbing S, Komiya A, McDonnell SK, Wiley KE, Jacobsen SJ, Isaacs SD, Walsh PC, Zheng SL, Chang BL, Friedrichsen DM, Stanford JL, Ostrander EA, Chinnaiyan AM, Rubin MA, Xu J, Thibodeau SN, Friedman SL, Martignetti JA. (2005) A germline DNA polymorphism enhances alternative splicing of the KLF6 tumor suppressor gene and is associated with increased prostate cancer risk. *Cancer Res.* 65:1213-22.
3. Debes JD, Yokomizo A, McDonnell SK, Hebbing SJ, Christensen GB, Cunningham JM, Jacobsen SJ, Tindall DJ, Liu W, Schaid DJ, Thibodeau SN. (2004) Glutathione-S-transferase P1 polymorphism I105V in familial and sporadic prostate cancer. *Cancer Genet Cytogenet.* 155:82-6.
4. Schaid DJ, Guenther JC, Christensen GB, Hebbing S, Rosenow C, Hilker CA, McDonnell SK, Cunningham JM, Slager SL, Blute ML, Thibodeau SN. (2004) Comparison of microsatellites versus single-nucleotide polymorphisms in a genome linkage screen for prostate cancer-susceptibility Loci. *Am J Hum Genet.* 75:948-65.
5. Stanford JL, McDonnell SK, Friedrichsen DM, Carlson EE, Kolb S, Deutsch K, Janer M, Hood L, Ostrander EA, Schaid DJ. (2006) Prostate cancer and genetic susceptibility: a genome scan incorporating disease aggressiveness. *Prostate.* 66:317-25.
6. Liu Z, Lu H, Shi H, Du Y, Yu J, Gu S, Chen X, Liu KJ, Hu, CAA (2005) PUMA overexpression induces ROS generation and proteosome-mediated stathmin degradation in colorectal cancer cells. *Cancer Res.* 65:1647-1654.

## Appendices

Letter of support and sample information from the new collaborator, Dr. Stephen Thibodeau, professor and Co-director, Molecular genetics laboratory, Vice Chair for research, Department of laboratory medicine and Pathology, Mayo Clinic, Rochester, MN

## Publications directly related to the project in the first year:

1. Liu Z, Lu H, Shi H, Du Y, Yu J, Gu S, Chen X, Liu KJ, Hu, CAA (2005) PUMA overexpression induces ROS generation and proteosome-mediated stathmin degradation in colorectal cancer cells. *Cancer Res.* 65:1647-1654.
2. Fu XS, Hu C.-A. A, Chen J, Wang J, Liu, KJR (2006) Cancer genomics, proteomics and clinical applications. In "Genomics Signal processing and Statistics."



200 First Street SW  
Rochester, Minnesota 55905  
507-284-2511

**Stephen N. Thibodeau, Ph.D.**  
Division of Laboratory Genetics  
507-284-9185 Fax 507-284-0670  
sthibodeau@mayo.edu

April 29, 2004

Chien-an Andy Hu, M.S., Ph.D.  
Assistant Professor  
Department of Biochemistry and Molecular Biology  
BMSB-258  
University of New Mexico, School of Medicine  
Albuquerque, NM 87131

Re: Provision of Biospecimens by Mayo to Chien-an Andy Hu

Dear Dr Hu,

This letter outlines Mayo Clinic Rochester ("Mayo") policies and the terms under which we agree to provide human materials ("Biospecimens") to you and your Institution. This letter creates a legal agreement between Mayo, you, and your Institution.

The Biospecimens you are receiving consist of 50 ng of DNA (10 ul, 5ng/ul) from 61 patients with familial prostate cancer and from 48 normal/tumor pairs from patients with sporadic prostate cancer. This material will only be used in your research regarding the analysis of the PUMA gene in hereditary and sporadic prostate cancer. This transfer of Biospecimens from Mayo to you has been approved by the Biospecimens Subcommittee of the Mayo Rochester Research Committee at its meeting on: 4-24-04.

These Biospecimens are owned by Mayo and are provided under bailment. They are being delivered in trust to you for the research purposes defined above and for the limited period of that research. Upon termination of the research described above and at the instructions of Mayo, you shall either return the Biospecimens to Mayo or destroy all unused portions of the material.

The Biospecimens, and any components thereof, may not be sold, assigned or transferred to any other party (other than researchers at your Institution working with you) without the express prior written consent of Mayo. You and your Institution will assume all responsibility for the safe use and handling of the Biospecimens by you and your employees, agents, and representatives after the materials are provided to you by Mayo. Mayo is not responsible for any claims arising from your acceptance and/or use of these materials



Chien-an Andy Hu, M.S., Ph.D

2

April 29, 2004

The Biospecimens must be restricted to research experimentation in compliance with applicable laws and regulations. If you use the Biospecimens in any way to develop potentially commercial products or procedures, then you must notify Mayo of that development. Ownership of inventions conceived, developed, or reduced to practice in the course of using the Biospecimens will be determined in accordance with U.S. laws governing inventorship.

**You and your Institution's acceptance of the terms identified above is acknowledged by your acceptance and use of the Biospecimens provided by Mayo with this letter. You must not use the Biospecimens until your Institution has taken all actions necessary to legally accept the policies and legal terms set forth above.**

Sincerely,



Stephen N. Thibodeau, Ph.D.

William H. Donner Professor

Co-Director, Molecular Genetics Laboratory

Vice Chair for Research, Department of Laboratory Medicine and Pathology

SNT/djj

10 uL at 5ng/uL

48 NT PAIRS FROM THE SPORADIC 55 NT PAIRS

|   | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| A | 13417 | 17971 | 13411 | 17325 | 17304 | 17903 | 13418 | 17970 | 13412 | 17324 | 17303 | 17902 |
| B | 17887 | 17959 | 17320 | 17308 | 17296 | 17943 | 17886 | 17958 | 17319 | 17307 | 17295 | 17942 |
| C | 17963 | 13987 | 17909 | 13459 | 17957 | 17327 | 17962 | 13986 | 17908 | 13460 | 17956 | 17326 |
| D | 17941 | 17945 | 17981 | 17885 | 17955 | 17916 | 17940 | 17944 | 17980 | 17884 | 17954 | 17915 |
| E | 17893 | 17927 | 17314 | 17891 | 17975 | 17899 | 17892 | 17926 | 17313 | 17890 | 17974 | 17898 |
| F | 13389 | 17330 | 13748 | 17889 | 17336 | 17939 | 13390 | 17328 | 13749 | 17888 | 17335 | 17938 |
| G | 17294 | 17334 | 17965 | 13399 | 17977 | 17947 | 17293 | 17333 | 17964 | 13400 | 17976 | 17946 |
| H | 17907 | 17912 | 17967 | 13453 | 17969 | 17885 | 17906 | 17925 | 17966 | 13454 | 17968 | 17894 |

TUMORS

NORMALS

The normal locations on the RIGHT half are paired with the tumors on the LEFT sides  
 example: 1A is paired with 7A

1B is paired with 7B

3C is paired with 9C

| 10 uL at 5ng/uL |       |       |       | Chromosome 19 families (bloods) |       |       |       |       |   |    |    |    |
|-----------------|-------|-------|-------|---------------------------------|-------|-------|-------|-------|---|----|----|----|
|                 | 1     | 2     | 3     | 4                               | 5     | 6     | 7     | 8     | 9 | 10 | 11 | 12 |
| A               | 36618 | 32354 | 35708 | 29356                           | 30499 | 32333 | 35859 | 54748 |   |    |    |    |
| B               | 46630 | 32167 | 36431 | 29810                           | 30320 | 32667 | 54917 | 46831 |   |    |    |    |
| C               | 45794 | 32537 | 36535 | 30071                           | 30322 | 32821 | 36498 | 47856 |   |    |    |    |
| D               | 29144 | 32469 | 37415 | 30075                           | 30460 | 32900 | 37570 | 31076 |   |    |    |    |
| E               | 29511 | 32485 | 47852 | 30080                           | 31832 | 33210 | 38363 | 31500 |   |    |    |    |
| F               | 29565 | 35530 | 45074 | 30193                           | 31242 | 33910 | 46689 |       |   |    |    |    |
| G               | 31921 | 34586 | 45438 | 30257                           | 32407 | 34800 | 45940 |       |   |    |    |    |
| H               | 32148 | 35528 | 29385 | 30256                           | 32104 | 35843 | 51768 |       |   |    |    |    |